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Research Article

The effect of under- and overexpressed *CoEXG1*-encoded exoglucanase secreted by *Candida oleophila* on the biocontrol of *Penicillium digitatum*

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Abstract

The yeast, *Candida oleophila*, is acknowledged for its biocontrol activity against postharvest moulds. However, the mechanism of this activity is not fully understood. One of the conjectured modes of action is associated with extracellular lytic enzymes, such as β-exoglucanase. The relationship of β-exoglucanase in the biocontrol activity of *C. oleophila* was investigated by generating *C. oleophila CoEXG1*-knockouts and double-*CoEXG1* transformants. The knockout transformants secreted 0–13% of the exoglucanase activity detected in the medium of the untransformed yeast (depending on the medium), indicating that *CoEXG1* is the main gene responsible for the production of the secreted exoglucanase. Correspondingly, the double-*CoEXG1* transformants secreted approximately twice as much 1,3-β-exoglucanase as the untransformed *C. oleophila*. The biocontrol activity of the *CoEXG1*-knockout and the double-*CoEXG1* transformants against *Penicillium digitatum* did not differ from that of the untransformed *C. oleophila* on kumquats. These results imply that the 1,3-β-exoglucanase encoded by the gene *CoEXG1* is not involved in the biocontrol activity of *C. oleophila* against *P. digitatum* under these experimental terms. However, these findings do not rule out the possibilities, that the participation of *CoEXG1* in biocontrol is dependent on the activity of other gene products, or that its effect may be manifested under altered environmental conditions.

Keywords: **yeast; *Candida*; biocontrol; exoglucanase**

Introduction

Exoglucanases are among the most abundant cell wall hydrolases in yeasts (Esteban *et al*., 1999). They have been purified and characterized from a number of yeast culture media and/or extracts, e.g. *Pichia polymorpha* (Villa *et al*., 1975), *Candida albicans* (Chambers *et al*., 1993a) and *Saccharomyces cerevisiae* (Nebreda *et al*., 1986, 1987). These enzymes seem to be necessary for the modification of β-glucan, a major structural component of the cell wall, during morphological processes such as budding, cell separation, mating and sporulation, as well as a nutritional role, such as in the hydrolysis of exogenous material for uptake (Larriba *et al*., 1995). In addition, in *C. albicans* this enzyme was found to have transferase, as well as hydrolytic activity (Stubbs *et al*., 1999).

Exoglucanase genes have been cloned from a number of yeasts, including *Kluyveromyces lactis* (Esteban *et al*., 1999), *C. albicans* (Chambers *et al*., 1993a) and *S. cerevisiae* (Nebreda *et al*., 1986). Recently, the 1,3-β-exoglucanase of *C. oleophila,* termed *CoEXG1,* has been cloned and expressed in *S. cerevisiae* (Segal *et al*., 2002).

The most intensive study concerning exoglucanases has been performed in *S. cerevisiae,* in which three exoglucanase genes have been

identified: *EXG1,* encoding for a polypeptide, whose heterogeneous glycosylation accounts for the two extracellular β-exoglucanases secreted into the medium (Nebreda *et al*., 1987); *EXG2,* encoding for a minor exoglucanase that remains attached to the cell wall (Nebreda *et al*., 1986) and *SSG1*, which encodes for a sporulation-specific 1,3-β-exoglucanase (del Rey *et al*., 1980). The open reading frame of *CoEXG1* of *C. oleophila* has been found to be similar to that of the *S. cerevisiae* exoglucanase gene, *EXG*1 (Segal *et al*., 2002).

C. oleophila has been shown to have biocontrol activity against postharvest decay of citrus fruits caused by *Penicillium italicum* and *P. digitatum* (Droby *et al*., 1998). However, unless it is supplemented with low levels of fungicides, it does not provide sufficient protection or curative activity against the pathogens (Droby *et al*., 1998). A prerequisite for improving the biocontrol activity of *C. oleophila*, or of other biocontrol yeasts in general, is the elucidation of their mode of action. There are reports demonstrating that the biocontrol activity may involve nutrient competition (Droby *et al*., 1989; Janisiewicz and Bors 1995; Castoria *et al*., 2001), site exclusion (Janisiewicz and Bors, 1995), induction of resistance (Droby *et al*., 2002), as well as direct parasitism, associated with the production of cell wall degrading enzymes, such as chitinase and 1,3-β-exoglucanase (Wisniewski *et al*., 1991; Cohen-Kupiec *et al*., 1999; Castoria *et al*., 2001; Masih and Paul, 2002). Both *Pichia guilliermondii* (Wisniewski *et al*., 1991) and *Pichia membranifaciens* (Masih and Paul, 2002) were found to exhibit high levels of β-1,3-glucanase activity, when cultured on various carbon sources or with cell walls of several fungi. Cells of these yeasts attached themselves to the mycelium of the mould, *Botrytis cinerea*, and caused partial degradation of the cell wall. There are indications that 1,3-β-glucanase of *Trichoderma harzianum*, a soil-borne filamentous fungus, is responsible for degrading cell walls of plant pathogenic fungi (Cohen-Kupiec *et al*., 1999). In experiments with *Aureobasidium pullulans*, a yeast antagonist of postharvest moulds, 1,3- β-glucanase activity was detected both *in vitro* and in apple wounds, the main site of fungal pathogen penetration (Castoria *et al*., 2001). In addition, 1,3-β-exoglucanase activity of two different antagonistic yeasts was higher in the culture filtrate of the more effective antagonist (Castoria *et al*., 1997).

In the present study a 1,3-β-exoglucanase enzyme secreted by *C. oleophila* was characterized and its role in the biocontrol of *P. digitatum* investigated by experiments involving the loss of function and over-expression of this enzyme.

Materials and methods

Strains and media

C. oleophila, strain I-182 (source of the commercial product Aspire™) and transformants were routinely cultured in YPD medium (1% yeast extract, 2% peptone, 2% D-glucose) or in potato dextrose broth (PDB) medium. For solid medium, 2% agar was added before autoclaving.

Plasmids

pUCARSHYG#, described in the article of Hara *et al*. (2000), was kindly provided by Mitsuyoshi Ueda. It contains a mutated hygromycin-B resistance gene, *HYG#*, in which all CTG codons in the open reading frame have been mutated to CTC. *HYG#* is under the control of the phosphoglycerate kinase (*PGK*) promoter with the *PGK* terminator at its 3′ end. Both the *PGK* promoter and *PGK* terminator are from *Candida tropicalis*.

The plasmid pGY278 was constructed from pRS426, a 5.7 kb yeast multicopy cloning vector (Christianson *et al*., 1992), and from two inserts. The inserts comprised: (a) a *CoEXG1* PCR product, which expresses 1,3-β-exoglucanase and has been cloned from *C. oleophila* (Segal *et al*., 2002); and (b) PHP, a PCR product of *HYG#* flanked by the *PGK* promoter and terminator. PHP served as the knockout-reporter gene. The *CoEXG1* PCR product was generated by PCR in a test tube containing 1 µl genomic DNA of *C. oleophila* and 20 pmols each of two primers, COEXG1A (GGGCTCGAGCCGAATATGGAGTGGTACAG), with a *Xho*I site at the 5′ end, and COEXG1B (GCGGGATCCATCGATGAAATAAATTATTTTATTGAAATTATGC) with *BamH*I at the 5′ end, to produce a 2509 bp segment, homologous to the 478–2962 bp portion of the *CoEXG1* sequence (Accession No. AF393806). This segment included the promotor, as well as the open reading frame, of the 1,3-β-exoglucanase gene. The PHP PCR was carried out with 0.01 µl pUCARSHYG# and 20 pmols

each of the primers specific to the promoter and terminator of *PGK*, respectively: 5′ primer (PGK3A–GGGACGCGTCTTAAGACATACGACATTTGTGCA), containing *Mlu*I and *BfrI*, in that order, at the 5′ end, and 3′ primer (PGK3B–GGGACGCGTACCAAGAATTGACGGCTG), with *Mlu*I at the 5′ end. This latter PCR produced a PCR fragment of *HYG#*, flanked by the promoter and terminator of *PGK*. Both the above PCR products were generated with the Expand™ High Fidelity PCR System (Roche) in reaction mixtures that included 2.5 units *Thermus aquaticus* (TAQ) polymerase and a 10× reaction buffer containing 15 mM $MgCl_2$, as well as 0.2 mM deoxynucleotides (dNTP). Reactions of 50 µl were executed at: 94 °C, 5 min; 50 °C, 2 min; and 72 °C, 1 min; followed by 29 cycles of 94 °C, 1 min; 50 °C, 2 min; and 72 °C, 3 min. In the final cycle, elongation was 5 min. The *CoEXG1* PCR product and pRS426 vector were digested with the enzymes, *Xho*I and *BamH*I, purified on S-400 columns (Pharmacia) and ligated, creating the pGY278 plasmid. In order to produce the pGY279 plasmid, pGY278 and the PHP PCR product were digested with *Bfr*I and *Mlu*I, purified on S-400 columns and ligated together, creating a plasmid that contained the *CoEXG1* exoglucanase gene with a 635 bp deletion (67 bp upstream and 568 bp downstream of the *CoEXG1* start codon), as well as a disruption caused by the insertion of PHP instead of the deleted sequence.

Transformants

The construction of the double-*CoEXG1* gene *C. oleophila* transformants has been described in a former article (Yehuda *et al*., 2001). Briefly, they were produced from the plasmid, pGY275, which was constructed from pRS426 (Christianson *et al*., 1992), and by two side-by-side inserts: (a) *HYG#* as the genetic marker; and (b) *CoEXG1*. This plasmid was digested by *Bfr*I at the sole site in *CoEXG1,* followed by electroporation into *C. oleophila* and integration at the homologous site.

The *CoEXG1* knockouts were generated by digesting the pGY279 plasmid with *Xho*I and *BamH*I, creating a segment of PHP, flanked by 1098 bp of the *CoEXG1* gene on the 5′ end and 752 bp of this gene on the 3′ end. This segment was electroporated into *C. oleophila,* allowing for the replacement of the normal *CoEXG1* gene by the deleted–disrupted version. Electroporation was carried out according to the *C. oleophila* transformation system based on hygromycin B selection, as described by Yehuda *et al*. (2001).

DNA isolation

Genomic DNA was isolated from *C. oleophila* according to the protocol described by Hoffman and Winston (1987). Plasmids were purified from *E. coli* with the Concert™ Rapid Plasmid Miniprep System kit (Gibco BRL).

PCR identification of knockouts

DNA samples from transformant colonies that grew on YPD plates, supplemented with 500 µg hygromycin B (Calbiochem)/ml, were analysed for the presence of *HYG#* and the deleted–disrupted version of *CoEXG1*. PCR analysis of the *HYG#* gene (described in Yehuda *et al*., 2001) produced a 1026 bp product of the *HYG#* open reading frame, if the gene was present, and no product, if it was not. PCR analysis of the deleted–disrupted version of *CoEXG1* yielded a PCR product approximating 4500 bp, in contrast to the normal version, which produced a PCR product of 2699 bp. The *CoEXG1*-PCR was executed with 20 pmols of each of two primers: (a) COEXG3A–5′-GGGACGCGTCTTAAGACATACGACATTTGTGCA, 32 bp upstream of the 5′ end of the sequence homologous to the primer COEXG1A; and (b) COEXG3B–5′-GGGACGCGTACCAAGAATTGACGGCTG, 190 bp downstream of the sequence homologous to the 3′ end of the primer COEXG1B. The Expand™ Long Template PCR System (Roche) was used for this PCR, and the mixture included 1 µl of the yeast DNA to be assayed, 0.2 mM dNTPs, a 10× reaction buffer 1 (with 17.5 mM $MgCl_2$) and the TAQ polymerase supplied with the system. The 25 µl reaction was carried out as follows: 94 °C, 5 min; 52 °C, 2 min; 68 °C, 6 min; followed by 34 cycles of (94 °C, 1 min; 52 °C, 2 min; 68 °C, 6 min). In the final cycle, elongation was 16 min.

Southern blot analysis

Genomic DNA of *C. oleophila* transformants (approximately 1 µg/transformant) was digested with either *Sal*I or *Bfr*I, and the fragments were

separated on a 0.8% agarose gel. The DNA was transferred by the standard capillary method and cross-linked to a positively charged nylon membrane (Amersham), as described by the manufacturer of the membrane. The DNA for the probe was acquired from a gel-purified PCR product (Concert™ Rapid Gel Extraction System) of *CoEXG1*, which was amplified from 1 µl of the plasmid, pGY275 (described in Yehuda *et al.*, 2001), with 10 pmols each of the primers 5′ primer–COEXG1A and the 3′ primer COEXG1B in a 50 µl reaction mixture that included 2.5 units *Thermus aquaticus* (TAQ) polymerase and a 10× reaction buffer containing 15 mM $MgCl_2$ (Promega), as well as 0.2 mM dNTPs. It was performed at: 94 °C, 5 min; 50 °C, 2 min; 74 °C, 1 min; followed by 29 cycles of (94 °C, 1 min; 50 °C, 2 min; 74 °C, 3 min), with a final elongation cycle of 15 min. The labelling of the probe with digoxigenin-11-dUTP and DNA hybridization were performed with the colorimetric DIG DNA Labeling and Detection Kit (Roche).

1,3-β-Exoglucanase activity assay

Exoglucanase assays were initiated by adding 1 ml 0.04% 4-methylumbelliferyl-β-D-glucoside (MUG; Sigma, Israel) in 100 mM sodium acetate buffer, pH 5.4, to 0.2 ml growth medium that had been separated from the cells and diluted with 0.8 ml water. The reaction was carried out at 37 °C for 30 min. Directly following the reaction, the test tubes were placed on ice and the liberated 4-methylumbelliferone was measured immediately by a spectrofluorimeter (excitation at 350 nm, emission at 440 nm). Units of activity were defined as nmoles 4-methylumbelliferone released/min.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of exoglucanase

Cultures of *C. oleophila* (10 ml) and its transformants were grown for 11 days in PDB at 200 rpm at 30 °C. Cells were removed from the medium by centrifugation of $3200 \times g$ at 4 °C for 10 min. Subsequently, the medium was centrifuged at $20\,000 \times g$ for 10 min at 4 °C and the supernatant concentrated approximately 10 times on VectaSpin Micro columns (Whatman). Equal volumes of concentrated samples were pipetted on a 12% resolving and 4% stacking gel of a discontinuous SDS–PAGE (Laemmli, 1970). Gels were stained with Coomassie blue. Band size estimations were executed with the Quantity One program, version 4.2.1 (BIORAD).

Spore germination assay

Cultures of *C. oleophila* and its transformants, 201 and 789, were grown in PDB for 9 days. After centrifugation of the cultures at $3200 \times g$, the three supernatants, as well as PDB (which served as a reference), were frozen at −20 °C, thawed, centrifuged at $3200 \times g$ (to eliminate the sediment), concentrated 10× on an Amicon polyethersulphone PM 10 filter (Millipore) and finally passed through a 0.2 µm sterile filter. These 10× concentrated culture media and the concentrated PDB medium were diluted in double dilutions with sterile distilled water. To 0.09 ml each dilution, 0.01 ml 5×10^5 *P. digitatum* spores/ml in 50% PDB were added. These samples were incubated for 24 h at 15 °C. At least 100 spores per sample were observed microscopically and counted to determine the percent of spore germination in the tested medium relative to that in the corresponding dilution of the PDB reference. The percentage inhibition was calculated as follows: $100 \times (A - B)/A$, where A, the reference, is the percentage of germinated spores in a specified dilution of PDB and B is the percentage of germinated spores in the tested medium of the same dilution. Each value is the average of three experiments.

Results and discussion

C. oleophila transformants containing a double copy of *CoEXG1*

Eight transformants were produced by introducing the *Bfr*I-linearized pGY275 plasmid, which included the *CoEXG1* gene, into *C. oleophila* by means of a hygromycin-B-based transformation system (Yehuda *et al.*, 2001). These same transformants were analysed on two Southern blots with a *CoEXG*1 probe, in order to confirm that *CoEXG*1 had become integrated at the homologous site (Figure 1). The genomic DNAs of each of the transformants and of the untransformed *C. oleophila*, were digested with either *Sal*I, which does not digest pGY275, or with *Bfr*I, which digests this plasmid only once, in the *CoEXG*1 gene. Therefore, the band pattern obtained for the

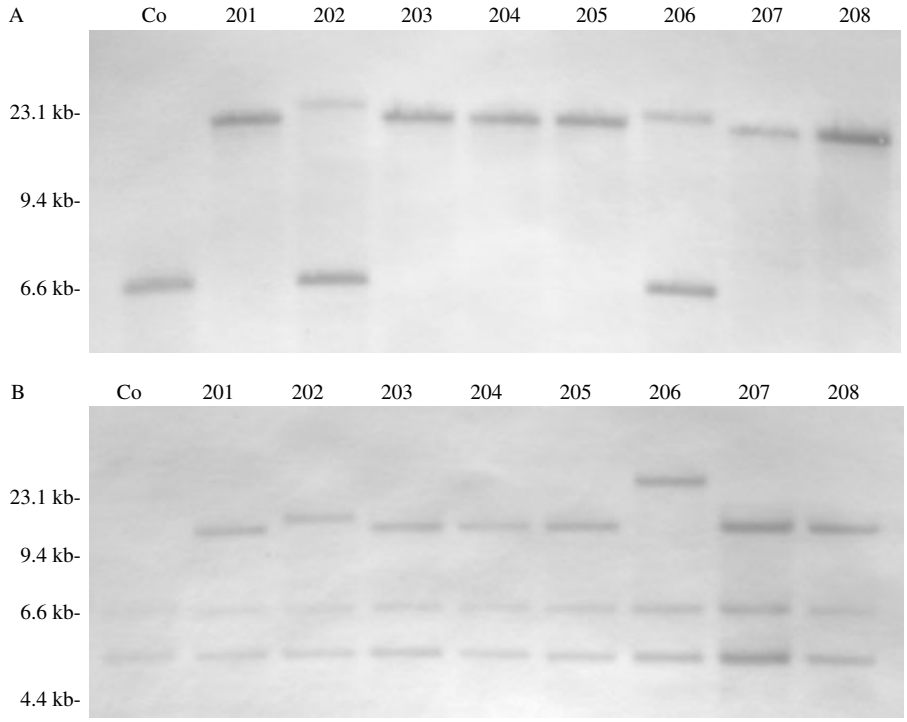

Figure 1. Southern blot analyses of genomic DNA from *C. oleophila* double-*CoEXG1* transformants. Co, untransformed *C. oleophila*; 201–208, transformants. The gel-purified PCR product of *CoEXG1* was used as the probe. (A) DNA digested by *Sal*I; (B) DNA digested by *Bfr*I

endogenous gene of the untransformed *C. oleophila* in the *Sal*I blot was a single band and in the *Bfr*I blot, a double band (Figures 1A and 1B, respectively). In both blots, there were no differences among the band patterns of the six transformants, 201, 203, 204, 205, 207 and 208. In the *Sal*I blot there was a single band of 17 kb for each of these transformants (Figure 1A). This size correlates with the band size that would be obtained when pGY275 (11 kb) is integrated into the endogenous *CoEXG1* gene of 6 kb. On the *Bfr*I blot there was a three-band pattern, comprising a ca. 11 kb band, which was the integrated plasmid, and two other bands of 7 kb and 5 kb, which were the segments located between the *Bfr*I site of the *CoEXG1* and the unidentified *Bfr*I sites upstream and downstream of it (Figure 1B). The latter two bands also appeared in the lane of *C. oleophila*. These data imply that these six transformants were products of integration by homologous recombination. The bands of the other two transformants, 202 and 206, included: (a) the probed endogenous segment of the native *CoEXG1* gene, as was detected in the lanes of the untransformed yeast; and (b) an additional band of varied length (longer than 11 kb), a result of random non-homologous integration of the plasmid.

The *Sal*I blot band pattern also indicated that *C. oleophila* is haploid. If *C. oleophila* was diploid, an additional band of 6 kb from the second allele would have been expected to appear in the lanes of the transformants, in which integration occurred by homologous recombination. The *Bfr*I Southern blot can not show the level of ploidity, since in this digest the haploid and the diploid would have the same band pattern.

Identification of *C. oleophila CoEXG1*-knockouts

After electroporation of *C. oleophila* with the digested pGY279 plasmid containing the deleted-disrupted *CoEXG1* gene and the *HYG#* gene (hygromycin B resistance genetic marker; see Materials and methods), colonies were picked, grown on YPD-hygromycin B plates and analysed for the presence of the *HYG#* gene by PCR.

Positive *HYG#* transformants were further analysed by PCR for *CoEXG1*. In order to determine whether replacement had taken place, primers that were homologous to the sequences located 32 bp upstream and 190 bp downstream of the site of expected homologous integration were employed. They were expected to produce a PCR fragment of 2.7 kb, if the endogenous *CoEXG1* remained unchanged and a ca. 4 kb fragment (2.7 kb, *CoEXG1*; and ca. 2 kb, PHP minus 635 bp deletion) when the deleted–disrupted *CoEXG1* gene had replaced the endogenous *CoEXG1* gene. Of the 70 colonies positive for hygromycin B resistance that were so analysed, only three were found to harbour the deleted–disrupted *CoEXG1* gene. Figure 2 shows results demonstrating the two PCR analyses. This very low rate of homologous recombination is in contrast to the 75% rate witnessed in the case of the pGY275 transformation (Yehuda *et al*., 2001). The reason behind this is most probably that the knockout transformation resulted from replacement, a process involving two sites of recombination on the genome, in contrast to recombination at a single site, occurring in the case of integration with the pGY275 in the double-copy transformation.

The fact that there is only one PCR product is in accordance with the haploidity of *C. oleophila,* which was implied in the Southern blot of the *Sal*I digest. If *C. oleophila* had been diploid, two bands would have been observed on the gel, one from the PCR of the chromosome that was transformed with the deletion–disruption fragment and the other from the PCR of the second allele, which had not been transformed.

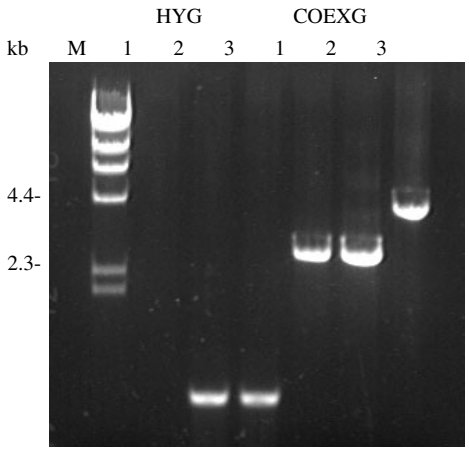

Figure 2. PCR analyses for *C. oleophila* transformants with *CoEXG1*-knockout. M, Lambda–*Hin*dIII; HYG–PCR products in *HYG#* analysis; COEXG, PCR products in *CoEXG1* analysis. 1, untransformed *C. oleophila*; 2, clone resulting from transformation, but not at the intended site; 3, the 789 *CoEXG1*-knockout clone resulting from transformation at the intended site

Since exoglucanases seem to be necessary for the modification of a major structural component of the cell wall (Larriba *et al*., 1995; Esteban *et al*., 1999), under- or overexpression of this enzyme in *C. oleophila* would be expected to have an effect on the growth rate of this yeast. Nevertheless, the growth rates of the *CoEXG1*-knockout, the double-*CoEXG1* gene transformant and the untransformed *C. oleophila* were the same, each with a generation time of approximately 90 min under optimal conditions. Similarly, manipulations of the exoglucanase genes of *C. albicans* and *S. cerevisiae* had no effect on their growth (Nebreda *et al*., 1986; Chambers *et al*., 1993b; del Mar Gonzalez *et al*., 1997).

Exoglucanase expression in *CoEXG1*-knockouts and double-*CoEXG1* transformants

The six double-copy transformants the two transformants with non-homologous integration (202 and 206) the three knockouts, the untransformed *C. oleophila* and a *C. oleophila-HYG#* transformant were tested for their exoglucanase secretion. They were grown for 18 h in YPD and their medium analysed for 1,3-β-exoglucanase activity. As revealed in Figure 3, the medium of the double-copy transformants had 1.7–2.2 units/1 $\times$ 10^8 cells of 1,3-β-exoglucanase activity, which was twice the activity measured in the medium of the *C. oleophila-HYG#* transformant and the untransformed yeast, both 0.8 units/1 $\times$ 10^8 cells. The *CoEXG1*-knockouts produced 0.05–0.13 units/1 $\times$ 10^8 cells, 13% of the controls. This demonstrates that *CoEXG1* is responsible for most of the exoglucanase secreted by *C. oleophila*. In the remaining experiments carried out in this study, the double-*CoEXG1* transformants were represented by the 201 clone and the *CoEXG1* knockouts by the 789 clone.

Identification of glucanase protein in medium

Further analysis of the secreted 1,3-β-exoglucanase was performed by SDS–PAGE. The strains were grown in PDB growth medium. Since this medium

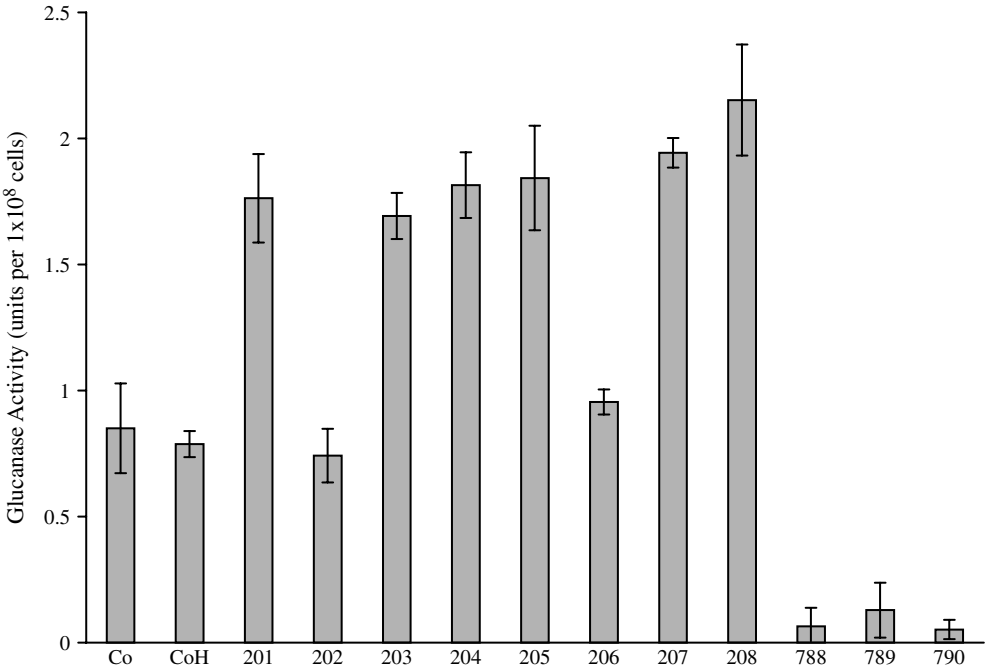

Figure 3. Glucanase secretion by *C. oleophila CoEXG1*-knockout and double-*CoEXG1* transformants. Strains were grown for 18 h in YPD at 30 °C and 200 rpm. Co, untransformed *C. oleophila*; CoH, *C. oleophila* transformed with *HYG#* alone; 201–208, double-*CoEXG1* transformants; 788–790, *CoEXG1*-knockouts. Columns represent averages of triplicates ± standard error

contains a negligible concentration of proteins, only those proteins secreted by the yeasts were observed on the gel (Figure 4). Equal volumes from PDB medium and from each of the 11-day PDB cultures: the untransformed *C. oleophila,* the *CoEXG1*-knockout transformant and the double-*CoEXG1* transformant, were purified, concentrated and run on an SDS–polyacrylamide gel. One major band was observed, and then only in the lanes of the untransformed *C. oleophila* and the double-gene transformant. The band in the double-gene transformant lane was thicker than that in the lane of the untransformed yeast, affirming that more enzyme was secreted by the double-*CoEXG1* transformant than by the untransformed *C. oleophila*. The size of the band was estimated to be 42 kDa (by the Quantity One Program) and is similar to the size calculated for the open reading frame of the *C. oleophila* gene, without the secretion signal peptide, 46 kDa. The molecular weight of the β-exoglucanase secreted by other yeasts was found to be 42 kDa in *C. albicans,* 43 kDa in *Candida parapsilosis*, 44 kDa in *Candida tropicalis*, 43, 47, 52 and 60 kDa in *Candida kefyr* and ca. 56 kDa for the major form and ca. 83 kDa for the minor form in *S. cerevisiae* (Nebreda *et al*., 1987; Chambers *et al*., 1993a). No band was detected in the lane of the knockout transformant, either because the amount of exoglucanase was too small or because there was none.

Another band of approximately 30 kDa, but barely visible on Coomassie-stained gels, accompanied the 42 kDa band. Like the 42 kDa peptide, it only appeared in the untransformed *C. oleophila* and double-*CoEXG1* transformant media. Therefore, it also seems to be a product of the *CoEXG1* gene. However, its significance has not been determined.

These SDS–PAGE results imply that the *CoEXG1*-knockout did not secrete exoglucanase when grown in PDB. This is in contrast to the data in YPD, in which it secreted 13% of that secreted by the untransformed *C. oleophila.* In order to verify whether the PAGE findings in PDB resulted from an undetectable amount of enzyme or from a lack of its production, the accumulation of exoglucanase secreted by the *CoEXG1*-knockout was recorded, and compared to that of the double-*CoEXG1* transformant and the untransformed *C. oleophila,* in PDB and YPD,

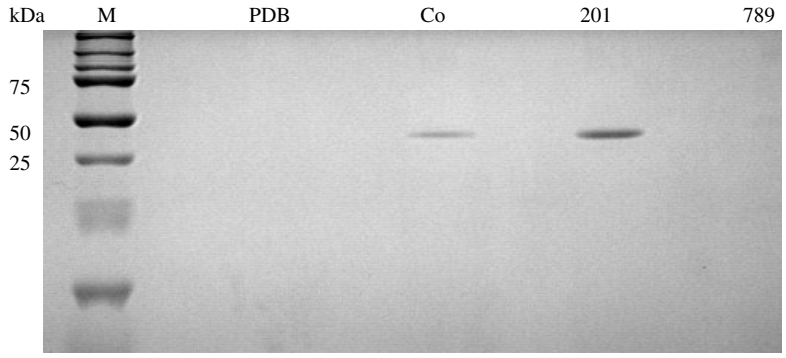

Figure 4. SDS–PAGE gel of glucanase in PDB cultures of *C. oleophila* and its transformants. Cultures were grown for 11 days. M, protein size marker (Precision Plus Protein Standards, Biorad); PDB, PDB alone; Co, untransformed *C. oleophila*; 201, double-*CoEXG1* transformant; 789, *CoEXG1*-knockout transformant. An equal volume of each sample was loaded in each well

over a 6 day period. The growth media used did not have a major influence on the glucanase secretion of the untransformed *C. oleophila* and its double-*CoEXG1* transformant. Both cultures secreted exoglucanase into both PDB and YPD media, with the double–*CoEXG1 C. oleophila* consistently secreting approximately twice as much exoglucanase as the untransformed yeast at each point measured. In contrast, the kind of medium had a noticeable effect on the glucanase secretion of the knockout. In YPD, knockout cultures secreted glucanase, with a small increase during the 6 days, while in PDB, the glucanase activity of the knockout cultures remained close to zero (Figure 5). These results hint at the existence of a second gene, whose secretion seems to be medium-dependent and whose secretion may be associated with an inducer/repressor mechanism. The *CoEXG1* gene seems to have a constitutive nature, in that its protein is secreted in both YPD and PDB.

Effect of 1,3-β-exoglucanase on spore germination of *P. digitatum*

In vitro experiments were executed in order to learn if the exoglucanase of *C. oleophila* had an effect on the germination of *P. digitatum* spores. The medium from 9 day-old PDB cultures of the untransformed *C. oleophila,* the double-*CoEXG1* transformant and the *CoEXG1*-knockout, as well as the PDB medium alone, were concentrated approximately 10 times and filter-sterilized (see Materials and methods). The concentrated PDB alone

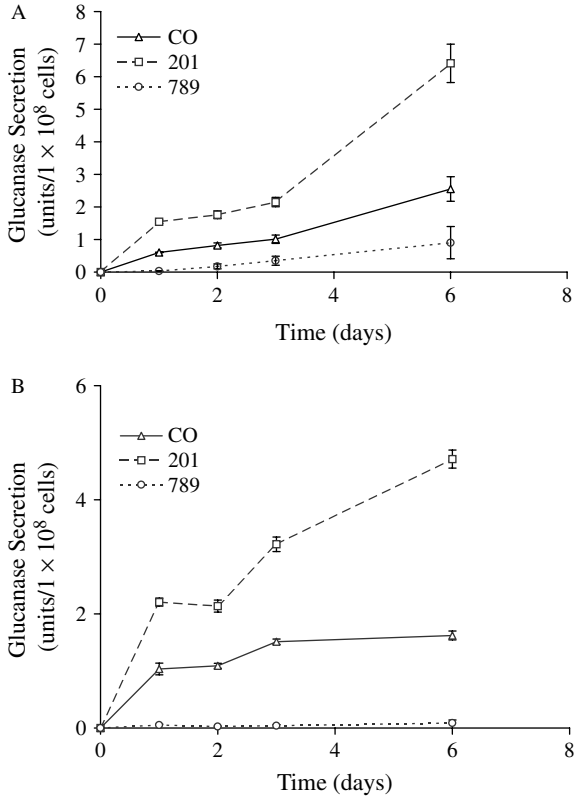

Figure 5. Exoglucanase secretion in two growth media. (A) YPD; (B) PDB. Co, untransformed *C. oleophila*; 201, double-*CoEXG1* transformant; 789, *CoEXG1*-knockout

was used as the reference medium for the maximum potential of spore germination. Glucanase activity (average of triplicates) in each was: PDB reference, 0 units/ml; untransformed *C. oleophila*,

46 units/ml; double-*CoEXG1* transformant, 175 units/ml; and the knockout, 2 units/ml. Spores of *P. digitatum* were added to the dilutions of the four different media that had been diluted by double dilutions of 1/1–1/16. As shown in Figure 6, there was no significant difference in the level of inhibition detected among the three media in which yeast had grown. However, all three media inhibited spore germination, as apparent by the correlation between the increase in medium dilution and the decrease in spore germination inhibition. According to these results, some product(s) was either secreted into, or possibly taken up from, the medium by *C. oleophila*, thus repressing the *P. digitatum* spore germination. However, the *CoEXG1*-encoded exoglucanase does not seem to be the inhibiting substance.

In vivo experiments were also carried out on untreated kumquats. Wounds in kumquats were treated with 1×10^7 cells of yeast, followed by infection with the 5×10^2 spores of the mould, *P. digitatum.* Three parameters of decay by the mould were surveyed, softness, hyphae growth and sporulation. In kumquats treated with the untransformed *C. oleophila*, the double-*CoEXG1* transformant and the CoEXG1-knockout, there was a delay of about 2 days in the appearance of decay, as compared with kumquats treated with *P. digitatum* alone. However, this same effect was also observed in the untransformed *C. oleophila.* These observations affirm the *in vitro* results, that the exoglucanase secreted by the *CoEXG1* gene is not essential for the biocontrol of *P. digitatum.* In any case, it can not be ruled out, that the *CoEXG1* product may react in an additive manner with other genes or is active in biocontrol under different conditions than those examined in this study.

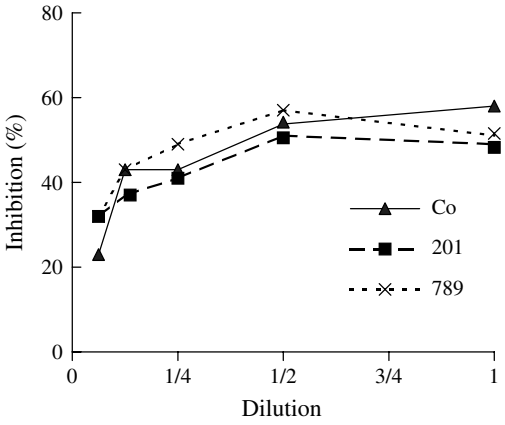

Figure 6. *In vitro* biocontrol assay of *P. digitatum* spore germination. Percentage inhibition of *P. digitatum* spore germination when germinated in concentrated media from 9 day-old cultures of: Co, untransformed *C. oleophila*; 201, double-*CoEXG1* transformant; and 789, *CoEXG1* knockout. The concentrated media were diluted in double dilutions of 1/1–1/16

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